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Developing a Novel Sulfoxide-containing MS-cleavable Homobifunctional Cysteine Reactive Cross-linker for Studying Protein-Protein Interactions

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Abstract

Cross-linking mass spectrometry (XL-MS) has become an emerging technology for defining protein-protein interactions (PPIs) and elucidating architectures of large protein complexes. Up to now, the most widely used cross-linking reagents target lysines. While such reagents have been successfully applied to map PPIs at the proteome-wide scale, comprehensive PPI profiling would require additional cross-linking chemistries. Cysteine is one of the most reactive amino acids and an attractive target for cross-linking owing to its unique role in protein structures. Although sulfhydryl-reactive cross-linkers are commercially available, their applications in XL-MS studies remain sparse–likely due to the difficulty in identifying cysteine cross-linked peptides. Previously, we have developed a new class of sulfoxide-containing MS-cleavable cross-linkers to enable fast and accurate identification of cross-linked peptides using multistage tandem mass spectrometry (MS^n). Here, we present the development of a new sulfoxide-containing MS-cleavable homobifunctional cysteine reactive cross-linker, **B**is**m**aleimide **S**ulf**o**xide (BMSO). We demonstrate that BMSO cross-linked peptides display the same characteristic fragmentation pattern during collision induced dissociation (CID) as other sulfoxide-containing MS-cleavable cross-linked peptides, thus permitting their simplified analysis and unambiguous identification by MS^n. Additionally, we show that BMSO can complement amine- and acidic residue- reactive reagents for mapping protein interaction regions. Collectively, this work not only enlarges the toolbox of MS-cleavable cross-linkers with diverse chemistries, but more importantly expands our capacity and capability of studying PPIs in general.

Graphical Abstract

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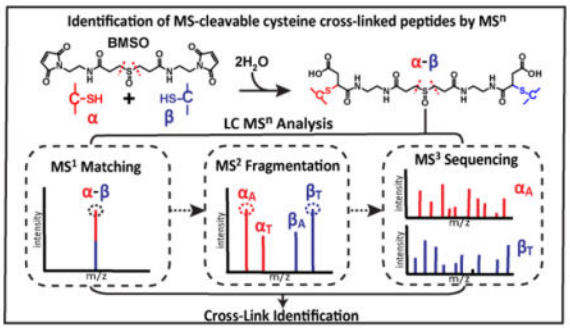

INTRODUCTION

Proteins rarely act alone in cells, instead, often functioning in the context of multi-subunit protein complexes. These macromolecular entities participate in complex protein-protein interaction (PPI) networks essential for controlling a diverse range of biological processes. Dysregulation of endogenous PPI networks can be detrimental to cell homeostasis and viability, which has been linked to multiple human diseases. Thus, protein interaction interfaces have become attractive targets for drug discovery [1,2]. Therefore, full characterization of PPIs can help not only understand the assembly, structure and function of protein complexes, but also identify PPIs critical in human pathologies, diagnostics and therapeutics.

Owing to recent technological innovations, cross-linking mass spectrometry (XL-MS) has become a powerful and effective method for studying PPIs *in vitro* and *in vivo* [3–7]. In comparison to other structural tools, XL-MS is unique due to its capability to simultaneously capture PPIs from their native environment and uncover their physical interaction contacts, thus permitting the determination of both identity and connectivity of PPIs in cells [3,6,7]. In addition, identified cross-links provide distance constraints to facilitate three-dimensional modeling of protein complexes by refining existing high-resolution protein structures and/or complementing low-resolution structures to elucidate architectures of large protein complexes [4,5,7] that have remained recalcitrant to traditional methodologies alone.

To date, amine-reactive reagents targeting lysine residues are still the most commonly used cross-linkers in XL-MS studies. This is due to the relatively high occurrence of lysines in protein sequences (~6% of all residues) and at surfaces of protein structures, as well as the specificity and efficiency of amine-reactive chemistries. However, it is clear that lysine-reactive reagents alone cannot provide a full picture of PPI maps as some protein interaction interfaces do not contain proximal lysines for cross-linking. Therefore, exploring additional cross-linking chemistries would be important for generating comprehensive PPI network topologies. Recently, acidic residue targeting reagents, i.e. non-cleavable [8] and MS-cleavable [9] dihydrazides have been developed and proven successful in yielding complementary information to lysine cross-linkers, thus expanding the coverage of PPI regions and aiding in protein structure characterization. Apart from lysines and acidic residues, cysteines are useful alternatives for protein cross-linking due to several factors. First, the high specificity and efficiency of sulfhydryl chemistry has permitted its widespread adoption in a gamut of

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proteomics studies. Secondly, cysteine cross-linking can be more selective and informative due to the lower prevalence of cysteines compared to lysine and acidic residues. Therefore, developing new cysteine-targeting cross-linkers would be advantageous for profiling PPIs, and can further complement existing reagents.

Among various types of cysteine-reactive reagents, maleimides are most widely utilized due to the specificity and efficiency of thiol-maleimide coupling [10]. In addition, maleimide moieties are also easily functionalized and reactions can occur at physiological pH in the absence of catalysts or heating, making these reagents well-suited for a variety of experimental uses. Such chemistry has been successfully employed in cross-linking studies to probe protein structures and identify interaction domains [10–12]. While homobifunctional maleimide cross-linkers are commercially available, their uses in XL-MS studies remain sparse. This is more likely due to difficulty in MS identification of cross-linked peptides. During the development of lysine and acidic residue-targeting reagents, it has been demonstrated that MS-cleavable cross-linkers are powerful and effective in facilitating accurate identification of cross-linked peptides [3,9,13–22]. In recent years, we have successfully developed a suite of sulfoxide-containing, MS-cleavable cross-linkers (i.e. DSSO (Figure 1A) [17], DMDSSO [19], Azide/Alkyne-A-DSBSO [20,23] and DHSO (Figure 1B) [9]) that permit simplified and accurate identification of cross-linked peptides. The MS-labile C-S bonds adjacent to the sulfoxide can be selectively and preferentially fragmented prior to peptide backbone cleavage upon collision-induced dissociation (CID), physically separating the peptides for individual sequencing [9,17,19,20]. Notably, this robust and predictable fragmentation occurs independent of cross-linking chemistry, peptide charge and peptide sequence. These unique characteristics enable straightforward and unambiguous identification of cross-linked peptides by MS^n analysis coupled with conventional database searching tools [9,17,19,20]. Sulfoxide-containing MS-cleavable cross-linkers have been successfully applied to not only study PPIs *in vitro* [17,24–30] and *in vivo* [20,27], but also to quantify structural dynamics of protein complexes [19,31]. Given the robustness of sulfoxide-based cleavability, we aimed to design, synthesize and characterize a novel sulfoxide-containing MS-cleavable homobifunctional cysteine linker, namely, BMSO (**B**is-**m**aleimide **s**ulf**o**xide) to facilitate the identification of cysteine cross-linked peptides. To the best of our knowledge, BMSO represents the first generation of cysteine-reactive cross-linking reagents with MS cleavability, which undoubtedly enhances our capability of mapping PPIs in the future.

EXPERIMENTAL PROCEDURES

Materials and Reagents

General chemicals were purchased from Fisher Scientific or VWR International. Bovine serum albumin (≥ 96% purity) was purchased from Sigma-Aldrich. Ac-LR9 peptide (Ac-LADVCAHER, 98% purity) was custom ordered from Biomatik (Wilmington, DE).

Synthesis and Characterization of BMSO

BMSO was synthesized as described in Figure 1D. Disuccinimidyl sulfoxide (DSSO) and the trifluoroacetate salt of 1-(2-aminoethyl) maleimide were synthesized as previously

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published [17,32] To a cooled (0 °C) solution of disuccinimidyl sulfoxide (468 mg, 1.21 mmol) and the maleimide salt (674 mg, 2.65 mmol) in H_2O (12 mL) was added 1M aq. $NaHCO_3$ (3.6 mL). After stirring for 12 h while allowing the reaction vessel to reach room temperature, the mixture was concentrated *in vacuo*. The crude material was then purified with column chromatography (30% MeOH in CH_2Cl_2) to afford BMSO as a colorless solid (251 mg, 47%): mp 90—98 °C; ^{1}H-NMR (600 MHz, $CDCl_3$) δ 6.76 (t, J = 5.5 Hz), 6.73 (s, 4H), 3.66—3.70 (app t, J = 5.7 Hz, 4H), 3.39—3.49 (m, 4H), 3.05—3.10 (m, 2H), 2.87—2.91 (m, 2H), 2.61 (app t, J = 7.1 Hz, 4H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ170.92, 170.35, 134.2, 46.9, 38.7, 37.5, 28.8; IR: 3307, 3086, 1693, 1645, 1543, 1173, 1024 cm^{-1}; HRMS (ESI) *m/z* $[M + Na]^+$ Calcd for $C_{18}H_{22}N_4O_7SNa$ 461.1107; Found 461.1119.

BMSO Cross-linking of Synthetic Peptides

Synthetic peptide Ac-LR9 was dissolved in DMSO to 1 mM and cross-linked with BMSO in a 1:1 molar ratio of peptide to cross-linker. The resulting samples were diluted to 10 pmol/μL in 3% ACN/2% formic acid prior to MS^n analysis.

Preparation of BMSO Cross-linked Bovine Serum Albumin

50 μL of 50 μM BSA in PBS buffer (pH 7.4) was reacted with BMSO in molar ratios of 1:50 and 1:100. The cross-linking reaction was initiated by adding BMSO to protein solutions, reacted for 2 h at 37°C. Cross-linked protein samples were subjected to SDS-PAGE followed by in-gel digestion prior to MS^n analysis [17] (Supplemental Methods).

Liquid Chromatography-Multistage Tandem Mass Spectrometry (LC-MS^n) Analysis

BMSO cross-linked Ac-LR9 was analyzed by LC-MS^n utilizing an Easy-nLC 1000 (Thermo Fisher, San Jose, CA) coupled on-line to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA) [17]. BMSO cross-linked peptides of BSA were analyzed by LC-MS^n utilizing a Dionex UltiMate™ 3000 (Thermo Fisher, San Jose, CA) coupled on-line to an Orbitrap Fusion™ Lumos™ mass spectrometer (Thermo Fisher, San Jose, CA). LC-MS^n data extraction and database searching for the identification of BMSO cross-linked peptides were performed similarly as previously described [17,30] (Supplemental Methods).

RESULTS AND DISCUSSION

Development of a Novel Sulfoxide-containing MS-cleavable Cysteine-reactive Cross-linker

In order to improve the identification of cysteine cross-linked peptides, we sought to create a novel MS-cleavable cysteine-reactive homobifunctional cross-linking reagent by integrating the MS-cleavability of sulfoxide-containing cross-linkers [9,17,19,20] with maleimide chemistry. This resulted in the development of BMSO (bismaleimide sulfoxide or 3,3′-sulfinylbis(N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)propanamide) (Figure 1C). This cross-linker consists of two maleimide functional groups for cross-linking cysteines connected by a spacer arm containing a central sulfoxide group with two symmetric MS-cleavable C-S bonds. As shown, BMSO contains the same type of MS-cleavable bonds as DSSO and DHSO (Figure 1A–B) [9,17]. The synthesis of BMSO only requires one additional amide bond forming step using DSSO as the starting material (Figure 1D). The core of DSSO is elaborated through the addition of the trifluoroacetate salt of 1-(2-aminoethyl)

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maleimide to install the cysteine-reactive moiety [32]. BMSO has a spacer arm of 24.2 Å, well within the distance range among cross-linking reagents that have been successfully applied for studying PPIs [3].

Predicted MS^2 Fragmentation of BMSO Cross-linked Peptides

Similar to other residue-specific cross-linkers, BMSO cross-linking is expected to result in three types of cross-linked peptides: dead-end (type 0), intra-link (type 1), and inter-link (type 2) modified peptides [33]. As inter-links (type 2) provide the most useful information pertaining to the relative spatial orientation of cross-linked cysteine residues, we have focused on the characterization of BMSO inter-linked peptides here. Figure 1E illustrates the thiol-maleimide coupling reaction between cysteine sulfhydryl (-SH) groups and the maleimide functional groups of BMSO, resulting in a closed-ring **s**uccin**i**midyl **t**hio**e**ther (SITE) bond by way of Michael addition [34]. The succinimidyl moiety of a SITE can then undergo irreversible hydrolysis in aqueous buffer, opening the ring to produce a stable **s**uccinamic **a**cid **t**hio**e**ther (SATE). To distinguish between these two forms, we have designated the closed-ring SITE inter-linked peptide as (α_c-β_c)—in which 'c' represents 'closed-ring SITE'—and the open-ring form of the same BMSO inter-linked peptide as (α_o-β_o), where 'o' represents 'open-ring SITE' (Figure 1E). The mass difference between the fully closed and opened forms of BMSO cross-linked peptides is equivalent to the mass of $2H_2O$ (+32 Da).

Regardless of the thioester structures (SITE or SATE) covalently linked to the cysteine residues, BMSO cross-linked peptides share the same MS-cleavable bonds as previously reported sulfoxide-containing cross-linked peptides and are thus expected to yield the same characteristic fragmentation patterns that enable cross-link identification by MS^n analysis [9,17,19,20]. To illustrate, Figure 1F displays the predicted MS^2 fragmentation of a BMSO interlinked heterodimer (α-β), with either SITE or SATE structures on cross-linked cysteines. Cleavage of either one of the two symmetric C-S bonds results in physical separation of the two cross-linked peptide constituents, resulting in the detection of peptide fragment ion pairs (i.e. α_A/β_S or α_S/β_A). These fragment peptides are modified either with alkene (A) or sulfenic acid (S) moieties, remnants of BMSO following collision-induced dissociation. As previously noted for other sulfoxide-containing cross-linkers [9,17,19,20], the sulfenic moiety typically undergoes dehydration to become a more stable and dominant unsaturated thiol (T) moiety (Figure S-1). Therefore, the fragmentation pairs for a BMSO cross-linked peptide α-β are expected to be α_A/β_T and α_T/β_A (Figure 1F). Such characteristic CID-triggered cross-link fragmentation has been proven unique and robust to sulfoxide-containing cross-linkers, independent of cross-linking chemistry, peptide sequence and charge [9,17,19,20]. The resulting MS^2 fragment ions represent single peptide chains that can be subjected to individual MS^3 analyses, permitting unambiguous identification of both cross-linked peptide sequences and cross-linking sites.

Characterization of BMSO Cross-linked Model Peptides by MS^n Analysis

To evaluate BMSO cross-linking and establish an analytical workflow for the identification of BMSO cross-linked peptides, we first performed BMSO cross-linking using a synthetic cysteine-containing peptide Ac-LR9 (Ac-LADVCAHER). Due to this reaction being

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performed in DMSO, the major inter-linked product detected in MS^1 was an Ac-LR9 homodimer with closed-ring SITE, i.e. (α_c-α_c) (*m/z* 637.7849^{4+}) (Figure 2A). MS^2 analysis of this BMSO inter-linked homodimer generated a pair of dominant fragment ions α_{Ac}/α_{Tc} (*m/z* $625.29^{2+}/641.27^{2+}$) as expected for BMSO inter-linked homodimers (Figure 2B). Subsequent MS^3 analyses of α_{Ac} (m/z 625.29^{2+}) and α_{Tc} (m/z 641.27^{2+}) fragment ions yielded series' of b and y sequencing ions identifying them as Ac-LADVC$_{Ac}$AHER and Ac-LADVC$_{Tc}$AHER, respectively (Figures S-2 A–B), verifying a cysteine-cysteine BMSO linkage between two separate Ac-LR9 peptides.

During LC-MS^n analysis, the fully open-ring Ac-LR9 homodimer cross-linked with BMSO was also detected, i.e. (α_o-α_o) (m/z 646.7885^{4+}) (Figure 2C). As expected, its MS^2 analysis yielded the expected characteristic fragment ion pair α_{Ao}/α_{To} (*m/z* $634.30^{2+}/650.28^{2+}$). MS^3 analyses of these two MS^2 fragments allowed unambiguous peptide identification as Ac-LADVC$_{Ao}$AHER and Ac-LADVC$_{To}$AHER, respectively (Figure S-3 A–B). These results demonstrate that the state of the ring structures attached to cross-linked cysteines do not interfere with the characteristic MS^2 fragmentation of BMSO cross-linked peptides and their subsequent identification by MS^n analysis.

Apart from the fully closed and open-ring forms of BMSO inter-linked Ac-LR9 homodimers, an additional ion (α_c-α_o) (m/z 642.2861^{4+}) was detected, representing a half-hydrolyzed product containing two cross-linked peptides, one with closed-ring and the other with open-ring structures attached to cysteines. Its MS^n analysis further confirms that BMSO cross-linked peptides can be readily identified independent of the ring structures on cross-linked cysteines (Figure S-4). Nonetheless, the existence of different forms of the same BMSO cross-linked peptide not only increases sample complexity, but also decreases the abundance of each particular cross-link. Therefore, it would be most desirable to obtain a single form of BMSO cross-linked peptides for MS^n analysis. To this end, we examined several experimental conditions to favor complete SITE hydrolysis and thus generate the most stable form of BMSO cross-linked products–open-ring SATE structures. Incubation of BMSO inter-linked Ac-LR9 in 25 mM ammonium bicarbonate buffer overnight at 37°C led to a nearly complete (98.7%) conversion of the fully closed-ring form (α_c-α_c) to the fully open-ring form (α_o-α_o) (Figure S-5 A–B). This indicates that SITE hydrolysis can be induced in order to minimize the heterogeneity of resulting cross-linked peptides. While BMSO cross-linking of standard peptides was carried out in DMSO, protein cross-linking and digestion are typically performed in physiological buffers. Therefore, we suspect that the majority of BMSO cross-linked products for protein samples may be in open-ring states. However, similar procedures were carried out in following experiments to ensure homogenous cross-linked products for MS^n analysis.

Identification of BMSO Cross-linked Peptides of BSA

To evaluate BMSO cross-linking of proteins, we used bovine serum albumin (BSA) as our model protein since it has been previously used for characterizing cross-linking studies [9]. Importantly, BSA contains 35 cysteines out of a total of 607 amino acid residues (5.76%), well above the average cysteine content in the proteome (~1.2%) (http://www.uniprot.org). The general procedures for optimizing BMSO cross-linking and MS^n analysis of BMSO

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cross-linked proteins are very similar to those described for other sulfoxide-containing cross-linkers [9,17,19,20]. Briefly, BMSO cross-linking of BSA was optimized by titrating various ratios of protein to cross-linker concentrations, temperature and reaction time. The resulting cross-linked proteins were separated by SDS-PAGE, digested and subjected to LC-MS^n analysis. Figure 3 displays an exemplary MS^n analysis of a BMSO inter-linked peptide of BSA (α_o-β_o) (*m/z* 719.5689^{4+}). MS^2 fragmentation of this inter-linked peptide resulted in the production of two characteristic peptide fragment pairs: α_{Ao}/β_{To} (*m/z* 614.29^{2+}/815.84^{2+}) and α_{To}/β_{Ao} (*m/z* 630.28^{2+}/799.86^{2+}) as predicted for BMSO inter-linked heterodimers (Figure 3B). Subsequent MS^3 analysis of α_{Ao} (*m/z* 614.29^{2+}) (Figure 3C) determined its sequence as SHC_{Ao}IAEVEK, in which the cysteine in the 3rd position from the N-terminus was modified with an open-ring alkene moiety. MS^3 analysis of β_{To} (m/z 815.84^{2+}) identified its sequence as YIC_{To}DNQDTISSK, with the cysteine in the 3rd position from the N-terminus carrying an open-ring unsaturated thiol moiety (Figure 3D). Collectively, the inter-linked peptide was identified as [^{286}YICDNQDTISSK^{298} cross-linked to ^{310}SHCIAEVEK^{318}], describing a fully open-ring inter-link formed between C289 and C312 of BSA.

Similarly, a total of 41 unique BMSO inter-linked peptides of BSA were identified by LC-MS^n analysis, representing 38 unique C-C linkages (Table S-1). It is noted that nearly all modified peptides comprising BMSO cross-links were identified in the open-ring SATE state–with the exception of CASIQK, which was identified frequently with a closed-ring SITE. We suspect that the free amine group of the N-terminal cysteine may react with the 5-member SITE ring to form a more stable 6-member ring without any mass change, thereby preventing SITE hydrolysis. These results demonstrate that BMSO cross-linking is effective and that the heterogeneity of cross-linked products can be controlled (in other words, SITE hydrolysis can be stabilized by conversion to SATE products). More importantly, these results prove that BMSO cross-linked peptides exhibit the characteristic MS^n fragmentation patterns as expected for all sulfoxide-containing MS-cleavable cross-linked peptides [9,17,19,20], thus enabling their simplified and accurate identification using the same MS^n workflow as previously established for sulfoxide-containing cross-linkers.

BMSO Cross-linking Maps of BSA

To examine the efficacy and interaction coverage of BMSO cross-linking on our model protein, we first derived a 2-D cross-linking map using the unique C-C linkages identified (Figure 4A). Considering the spacer arm length of BMSO (24.2 Å) and the distances contributed by cysteine side chains (2.8 Å), as well as backbone flexibility and structural dynamics, we estimated that the theoretical upper limit for the Cα-Cα distances between BMSO cross-linked cysteine residues is ~45 Å. To determine whether the identified BMSO cross-links correlate to residues with distances below the theoretical limit, we mapped them onto a published BSA crystal structure (PDB: 4F5S). All identified cross-links were able to be mapped to the crystal structure (Figure 4B), with 97.4% (37 out of 38) having measurable Cα-Cα distances below 45 Å (Figure 4C, Table S-1). This indicates that nearly all cross-links satisfy the expected distance constraints permitted by the molecular structure of BMSO and that the captured cross-links correlate well with known BSA structure. In summary, our

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results suggest that BMSO cross-linking is effective for mapping protein-protein interactions.

Comparison of BMSO with DSSO and DHSO Cross-linking

To determine the complementarity of cysteine-reactive cross-linking with our previously developed amine-reactive and acidic residue-reactive cross-linkers, we have compared the cross-links identified using BMSO in this study to those previously reported using DSSO and DHSO [9]. As aspartic/glutamic acid residues are most abundant in BSA, it is not surprising that acidic-residue cross-linking yielded the highest number of cross-links overall (69). Interestingly, although BSA has more lysines (9.8%) than cysteines (5.7%), we have identified more C-C linkages than K-K linkages (i.e. 43 vs. 33, respectively). Compared to XL-MS maps derived from DSSO and DHSO cross-linking data [9], BMSO cross-linking improves the overall coverage by identifying proximal regions unfavored by amine- and acidic residue-targeting cross-linkers, thereby complementing previous results. This observation is most evident when examining the spatial relationships of the centrally located helices (H4, H13, H17, and H22) relative to more peripheral helices in the 3-D structure of BSA (PDB: 4F5S). These regions poorly covered by DSSO and DHSO are better characterized by BMSO, which has identified a total of 17 C-C-linkages describing clusters of physical contacts between: 1) H4 with H10 (C99-C200, C99-C223) and H17 (C99-C312); 2) H13 with H7 (C288-C147), H10 (C288-C200, C288-C223), H22 (C288-C392), H26 (C288-C471, C288-C484), and H28 (C288-C510); 3) H17 with H6 (C312-C125); and 4) H22 with H10 (C392-C200), H21 (C392-C383, C392-C384), H26 (C392-C471; C392-C484), and H28 (C392-C510). The number of cysteines and their positions within the core as well as the length of BMSO are likely contributing factors that enable the detection of cross-links within these regions. A large portion of identified cysteine cross-links within these clusters correspond to residues with Cα-Cα distances over 30 Å (11/17, 64.7%), which are more likely to be missed with cross-linkers containing shorter spacer arms (i.e. DSSO and DHSO). While BMSO appears to obtain more contacts in the center regions of BSA, DSSO and DHSO cross-linking have provided broader coverage of various regions, including both termini. This observation may also be correlated to the relative distribution of cysteines within the primary sequence and their roles in stabilizing the structure of BSA. While BSA is a single protein, our data suggests that the three distinct cross-linking chemistries can indeed facilitate a more comprehensive mapping of intramolecular BSA contacts. Therefore, we anticipate that such a combinatory XL-MS approach would be even more beneficial for detailed PPI profiling when applied to more complex samples such as large multiprotein assemblies and cell lysates.

CONCLUSION

Here we report the development and characterization of a novel sulfoxide-containing MS-cleavable cysteine reactive cross-linker, BMSO, derived from DSSO [17]. Using both a standard peptide and protein, we have demonstrated that BMSO cross-linking is efficient and that BMSO cross-linked peptides display the same characteristic MS-cleavability unique to other sulfoxide-containing cross-linked peptides [9,17,19,20]. Our results further illustrate that the same MS^n workflow can be applied for simplified and accurate identification of all

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sulfoxide-containing MS-cleavable cross-linked peptides regardless of cross-linking chemistries, thus facilitating rapid and simplified identification for BMSO cross-linked cysteine residues. However, unlike DSSO (NHS, amine-reactive) and DHSO (hydrazide, acidic residue-reactive) cross-linking chemistries, conjugation of cysteines via maleimide chemistry can yield two different forms of cross-linked cysteines, either containing the SITE (closed-ring) or hydrolyzed SATE (open-ring) structures (Figure 1E–F). While this occurrence potentially decreases cross-link spectral abundance, we have shown that SATE formation can be pushed near completion during the experimental process—minimizing cross-linked peptide ion heterogeneity during MS^n analysis. More importantly, the ring states of BMSO cross-linked cysteines do not interfere with their identification by MS^n analysis, permitting the identification of cross-links in regions not covered by and complementing the structural data afforded by lysine- and acidic residue-targeting cross-linkers.. To the best of our knowledge, BMSO represents the first MS-cleavable cysteine-reactive cross-linking reagent, and we expect that it will significantly advance cross-linking studies targeting cysteine residues—which are currently underrepresented in XL-MS analysis. The comparison showing the complementary nature of BMSO, DSSO and DHSO cross-linking data further signifies the necessity and usefulness of multiple cross-linking chemistries to obtain high-density interaction maps with improved confidence, which will undoubtedly expand our capacity and capability of mapping PPIs at the systems-level in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

XL-MS	cross-linking mass spectrometry
BMSO	Bismaleimide Sulfoxide, a.k.a. 3,3′-sulfinylbis(N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)propanamide
DSSO	Disuccinimidyl Sulfoxide
DHSO	Dihydrazide Sulfoxide
DMDSSO	Dimethyl Disuccinimidyl Sulfoxide
Azide/Alkyne-A-DSBSO	Azide/Alkyne-tagged, Acid-cleavable Disuccinimidyl Bis-sulfoxide
MS	mass spectrometry

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MS^n	multi-stage tandem mass spectrometry
CID	collisional induced dissociation
LC-MS^n	liquid chromatography multistage tandem mass spectrometry
SITE	succinimidyl thioether
SATE	succinamic acid thioether

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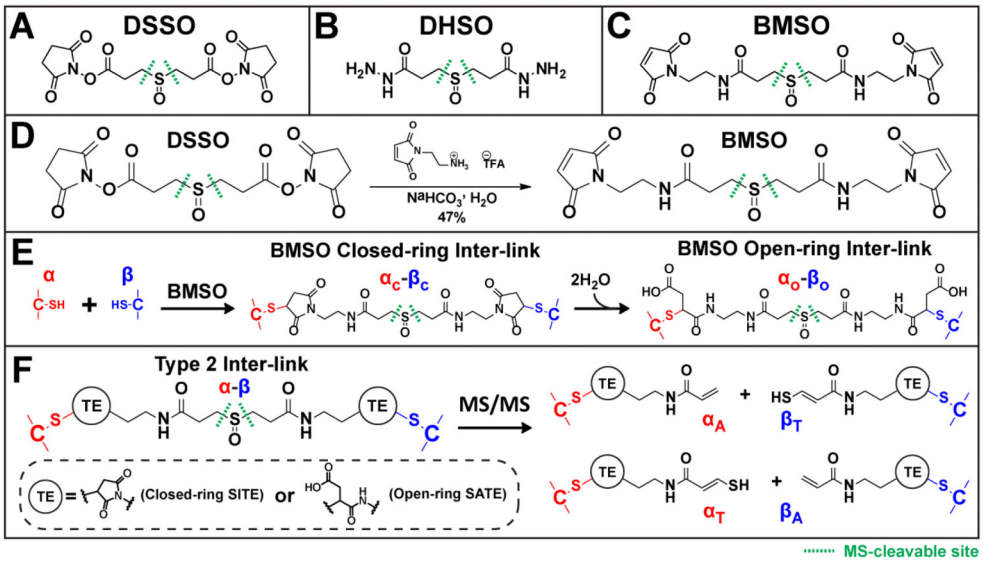

Figure 1. Design, synthesis and characteristics of the Sulfoxide-containing MS-cleavable Cysteine Reactive Cross-linker, BMSO

Molecular structures of (A) DSSO [17], (B) DHSO [9], and (C) BMSO. (D) Synthesis scheme of BMSO. (E) BMSO cross-linking results in the formation of an inter-linked heterodimer (α-β), in which the closed-ring SITE structure on cross-linked cysteines can be converted to open-ring SATE structures upon hydrolysis. (F) Predicted characteristic MS^2 fragmentation of a BMSO inter-linked heterodimer α-β, in which thioester (TE) structures on cross-linked cysteines can be in the form of either closed-ring SITE or open-ring SATE. Note: c: closed-ring; o: open-ring.

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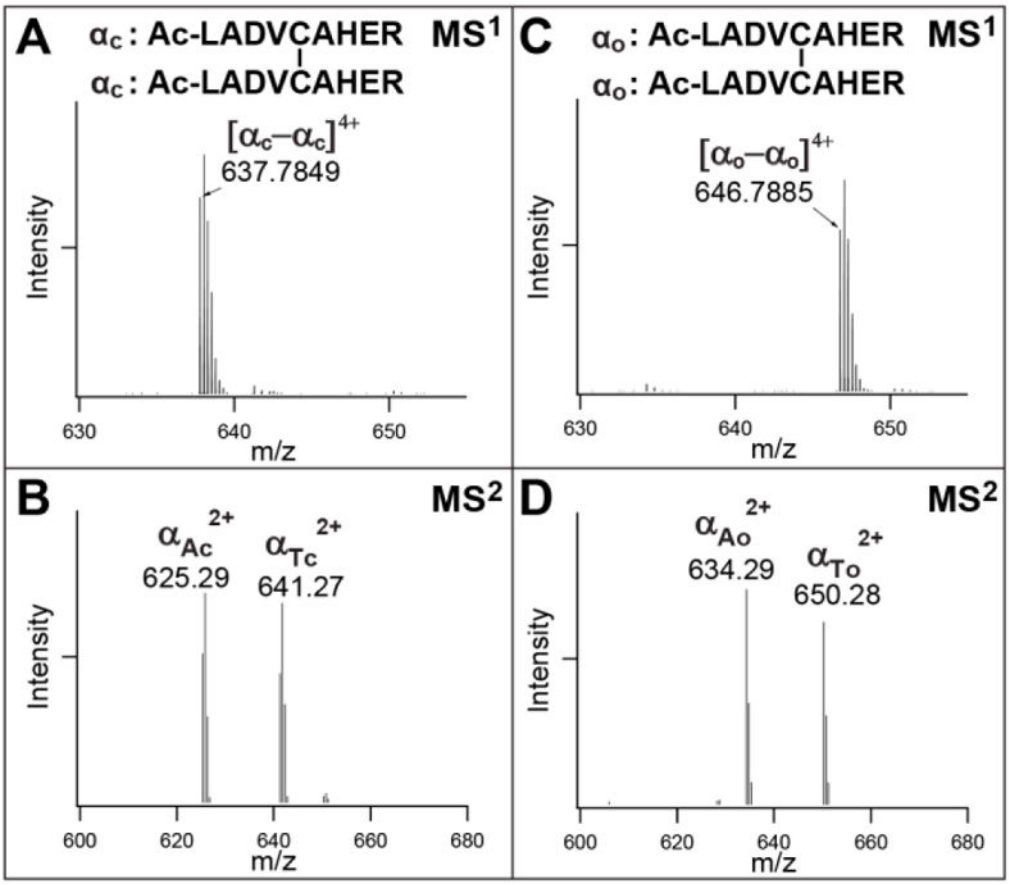

Figure 2. MS analysis of the BMSO inter-linked Ac-LR9 homodimer (α–α)

(A) MS^1 spectrum of the inter-link with closed-ring form, $(\alpha_c–\alpha_c)^{4+}$ (m/z 637.7849^{4+}). (B) MS^2 spectrum of the $(\alpha_c–\alpha_c)^{4+}$ detected in (A), in which two dominant fragment ions, i.e. α_{Ac} (m/z 625.29^{2+}) and α_{Tc} (m/z 641.27^{2+}), were detected as predicted for homodimer inter-links. (C) MS^1 spectrum of the inter-link with open-ring form, $(\alpha_o–\alpha_o)^{4+}$ (m/z 646.7885^{4+}). (D) MS^2 spectrum of the $(\alpha_o–\alpha_o)^{4+}$ detected in (C), in which two dominant fragment ions, i.e. α_{Ao} (m/z 634.29^{2+}) and α_{To} (m/z 650.28^{2+}) were detected as expected. Note: c: closed-ring; o: open-ring; Ac/Tc: alkene/unsaturated thiol moieties with closed-ring SITE; Ao/To: alkene/unsaturated thiol moieties with open-ring SATE.

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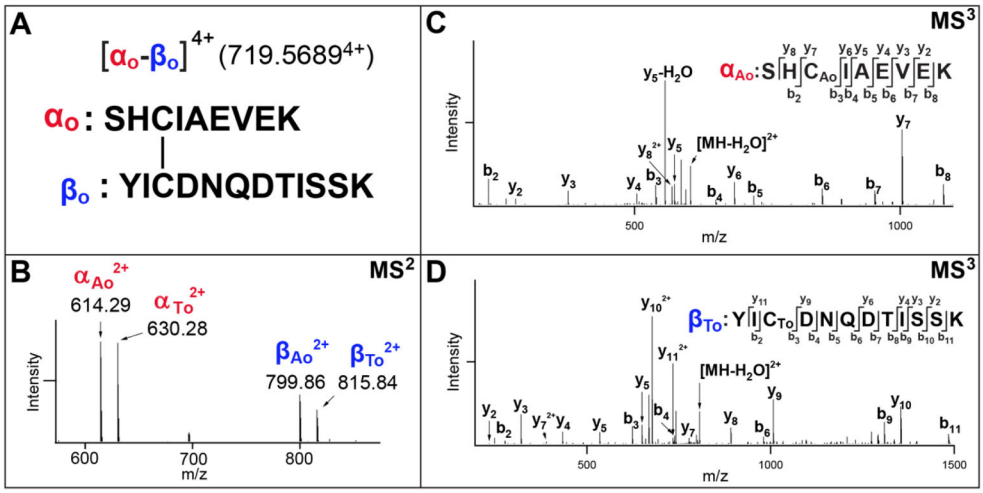

Figure 3. MS^n analysis of a representative BMSO inter-linked peptide of BSA

(A) The BMSO inter-linked peptide with open-ring SATE structures, (α_o–β_o) (m/z 719.5689^{4+}). (B) MS^2 spectrum of the (α_o–β_o), in which two characteristic fragment ion pairs were detected, i.e. α_{Ao}/β_{To} (m/z 614.29^{2+}/815.84^{2+}) and α_{To}/β_{Ao} (m/z 630.28^{2+}/799.86^{2+}). (C) MS^3 analysis of α_{Ao} (m/z 614.29^{2+}) identified the sequence as SHC$_{Ao}$IAEVEK, in which the cysteine residue was modified with alkene moiety carrying an open-ring SATE. (D) MS^3 analysis of β_{To} (m/z 815.84^{2+}) identified the sequence as YIC$_{To}$DNQDTISSK, in which the cysteine residue was modified with unsaturated thiol moiety carrying an open-ring SATE.

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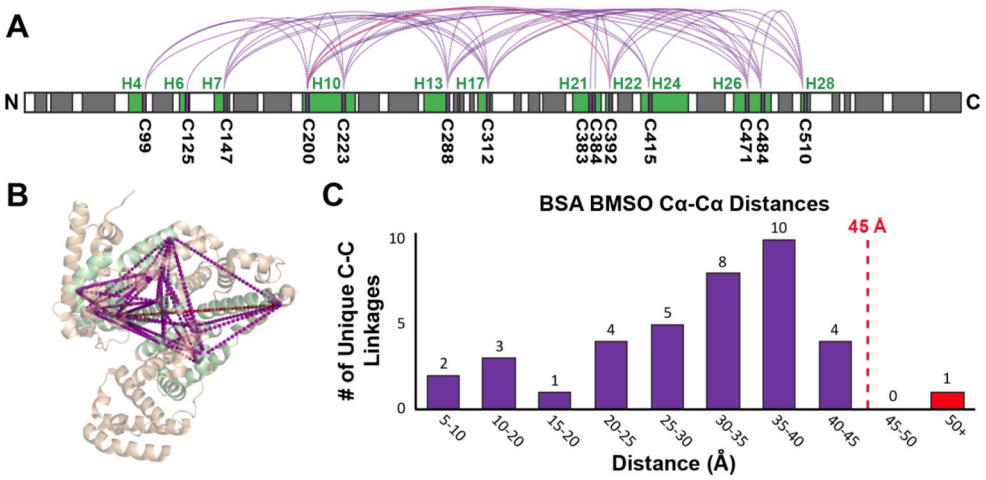

Figure 4. BMSO XL-MAP of BSA

(A) 2-D BMSO XL-map on BSA linear sequence. Helical secondary structures are designated by gray regions, green if containing cross-linked cysteines. (B) 3-D BMSO cross-link map on BSA crystal structure (PDB: 4F5S). Helices containing cross-linked cysteines are shown in green. (C) The distribution plot of identified C-C linkages vs. their spatial distances showing the number of linkages within (purple) and greater than (red) the expected distance constraint (< 45Å).